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ELASTASE IS A CONSTITUENT PRODUCT OF T CELLS

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SUMMARY: Proteases produced by immune cells have been found to be important components of the immune response to antigen. A protease previously unrecognized as a specific T cell product has been identified which has the gene sequence, serologic crossreactivity, and enzymatic specificity of elastase. T cell elastase, found in combination with the natural elastase inhibitor α_1-antitrypsin (α_1-protease inhibitor, α_1-PI), is produced by both $CD4^+$ and $CD8^+$ T lymphocytes, and is found both in a membrane-bound and in a soluble form in murine T cell lines.

INTRODUCTION: In an attempt to achieve functional analysis of the antigen-specific activity of T cells, a non-TcR mouse gene was identified in a T hybridoma (1). Sequence analysis of the cDNA clone of this mouse gene revealed it to have 86% homology with rat pancreatic preproelastase I (1). However, in experiments with porcine pancreatic elastase these authors were unable to demonstrate an elastase effect in the function of their hybridoma, nor could they demonstrate the presence of elastolytic activity in their T cell hybridoma products. We report here the proteolytic activity of elastase in a complex purified from both $CD4^+$ and $CD8^+$ T hybridomas.

MATERIALS AND METHODS:

Antibodies. Monoclonal antibodies 14-12 (2) and 14-30 (3) have been extensively characterized. These IgM antibodies recognize immunoregulatory factors of effector ($CD8^+$) and inducer ($CD4^+$) T cells, respectively. Sheep anti-human leukocyte elastase (Biodesign, Inc., Kennebunkport, ME) is immunoglobulin fraction. Anti-human α_1-PI (Boehringer Mannheim) is in the form of an antiserum.

Affinity purification of elastase complexes. Hybridomas were grown in RPMI-1640 supplemented with 10% fetal calf serum. In some cases, hybridomas were cultured in the

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presence of the elastase substrate N-t-Boc-L-Alanyl-Prolyl-Nvaline p-chloro thiobenzyl ester (4, BAPV, Sigma). Alternatively, T cell elastase complexes were purified from membrane extracted material as previously described (5). T cell elastase from the A1.1 T helper hybridoma (6), a generous gift from D. Green, La Jolla Institute for Allergy and Immunology, La Jolla, CA, had been affinity purified on antibodies to the α-chain of the T cell antigen receptor or on poly-18, the immuizing antigen. T cell elastase complexes from AF5.C6 (7), F12.23 (8), 51H7D (9), or Ts1-29 (10) T inducer hybridomas and from D10.G4.1 (11) T helper hybridoma were affinity purified from three day hybridoma culture supernatants utilizing the 14-30 mAb conjugated to CNBr-Sepharose. In addition, 34s-704 (12) was affinity purified on keyhole limpet hemocyanin (KLH), the immunizing antigen (5). T cell elastase from the Ts3-3KS-21 (13) T effector hybridoma was affinity purified utilizing the 14-12 mAb conjugated to CNBr-Sepharose. Culture supernatant (50 ml) or solubilized membrane (4 ml) were applied to columns (5 ml) which had been pre-equilibrated in 0.3M borate buffer, pH 8.6. After washing unbound material from the columns with five column volumes of borate buffer, bound material was eluted with 2 ml 0.2M Na_2CO_3, pH >12, collected in 25 ml borate buffer, dialyzed versus DH_2O, and lyophylized. This material was reconstituted in 50-100ul DH_20.

Elastolytic activity. T cell elastase complexes (15 ul) were diluted in wells of a polystyrene microtitre plate (Immulon, Dynatech Lab., Alexandria, VA). Elastase substrate was then added and elastolytic activity was measured at 405nm at various time points. Substrate consisted of 5 ul succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (SA^3NA, 25 mg/ml) diluted into 0.29 ml 0.2M Tris, pH 8.2. Porcine pancreatic elastase (Boehringer Mannheim, Indianapolis, IN) and human leukocyte elastase (Sigma) were used as controls. In some cases, in order to demonstrate elastolytic activity, T cell elastase was pre-incubated for 2 min at 37°C with porcine pancreatic trypsin (1ul of 2.5%, ICN Pharmaceuticals, Cleveland, Ohio), the only known activator of proelastase. Trypsin used for this purpose was previously inactivated for contaminating elastase with 25mg/ml methoxy succinyl L-Ala-L-Ala-L-Pro-L-Val chloromethylketone (Bachem, Torrance, CA), and excess inhibitor was removed by dialysis.

ELISA. Porcine pancreatic elastase (Boehringer Mannheim) was diluted with beginning concentration 1.25 ug/ml. T cell elastase complexes from 51H7D, AF5.C6, Ts1-29, and 34s-704 were used at 0.5 mg/ml. Primary antibody consisted of 14-12, 14-30 (1/100), or sheep anti-human leukocyte elastase (1/50). Binding of primary antibody was detected by incubating wells for 1 h at 23°C with horse radish peroxidase conjugated to protein A or protein G (1/500, Zymed). In the case of 14-12 and 14-30 which are IgM monoclonal antibodies, binding was visualized with monoclonal anti-mu chain conjugated with horse radish peroxidase (1/500, Zymed). After extensive washing in PBS-Tween, substrate was added consisting of 0.03% ABTS (2,2-azino-di-3-ethylbenzthiazoline-6-sulfonate) in 0.1M citrate buffer, pH 4.0 with 0.05% H_2O_2. Reactions were monitored at 415 nm.

SDS-PAGE and Western Blot analysis. Electrophoresis on 0.75 mm 12% polyacrylamide gels was performed using standard SDS-polyacrylamide gel electrophoresis (SDS-PAGE) buffers in reducing conditions, and transfer of proteins to Immobilon (Millipore Corp., Bedford, MA) was performed by electrophoresis of proteins in 0.025M Tris, 0.193M glycine, 20% MeOH. After blocking, Immobilon was incubated 1 h at 23°C with primary antibody consisting either of anti-human α_1-PI (1/50, Boehringer Mannheim) or sheep anti-human leukocyte elastase (1/50, Biodesign). Binding was detected by incubation of Immobilon with protein G-conjugated alkaline phosphatase (1/100, Zymed). After extensive washing, substrate was added consisting of 0.033% p-nitroblue tetrazolium chloride and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate in 0.1M diethanolamine, pH 8.0, containing 1 mM $MgCl_2$. Preparative polyacrylamide gels were sliced, individual slices were chopped fine, and material was allowed to leach from these fractions by incubating 4 h at 4°C in 5 ml PBS followed by dialysis of the supernatant overnight versus PBS. Extracted material was used in an assay measuring elastolytic activity.

In vitro translation of RNA. Total RNA was isolated using the guanidinium thiocyanate method and centrifugation through $CsCl_2$ (14). Poly A+ RNA was purified from total RNA by passage over an oligo(dt)-Sepharose column (14). Poly A+ RNA (1ug) was translated in vitro using wheat germ extract (Promega, Madison, WI) as recommended by the manufacturer.

PCR analysis of T cell hybridomas. Murine T cell lines used for PCR were Ts1-29, A1.1, and AF5.C6. RNA was prepared, added to 4ul 20% SDS and 2.5ul of 20 mg/ml proteinase K. Extractions were performed with an equal volume of phenol/chloroform/isoamyl alcohol twice and with chloroform/isoamyl alcohol once. The RNA was precipitated with ethanol and 0.3M sodium acetate, pelleted, and resuspended in water. Reverse transcription was achieved with BRL Moloney Murine Leukemia Virus reverse transcriptase. Assay conditions followed those recommended by BRL.

Oligonucleotide primers for Ts1-29 and Af5.C6 were designed containing 28 nucleotides for amplifying 726 bases of the previously described elastase gene (1). Hind III sites were engineered into the primers so that PCR products could be cloned into M13 for later sequencing. Primers used for PCR were as follows:

5'primer ACG TAA GCT TTC ATC CGA AGC AAC TGG G
3'primer ACG TAA GCT TTC GGC CAC TGA GCG ATA A.

An additional set of primers were used with A1.1 which contained 21 nucleotides for amplifying 417 bases of the same gene and were as follows:

5'primer CAT CTC AGA ATT CCC TCC AGT A
3'primer GGA ATT CTG CAG GGT CTG GGC C.

Each PCR reaction contained: 0.5M KCL, 0.01M Tris, pH 8.3, 1.5mM $MgCl_2$, 0.01% gelatin, 2mM dNTP's, 1ng of plasmid template, 100 pmoles of each oligonucleotide primer and 0.025U/ul of Taq polymerase (Cetus). Each PCR cycle was designed to consist of 10 sec. denaturation at 94°C, 2.5 min annealing at 55°C, and 2 min extension at 72°C.

RESULTS: In order to identify the components of a biologically active complex in T cells, material was first purified from $CD4^+$ inducer T hybridomas or from $CD8^+$ effector T hybridomas by affinity chromatography utilizing monoclonal antibodies 14-30 (3) or 14-12 (2) which have been previously characterized to be immunoreactive with antigen-specific factors of $CD4^+$ or $CD8^+$ T cells, respectively. By incubation of synthetic elastase-specific substrates (15) with purified material, the presence of elastase specific activity was then demonstrated (Fig. 1). Elastolytic activity of T cell elastase could be detected only after incubation over a period of several days or after co-incubation with trypsin, the only known naturally occurring activator of proelastase (16).

Commercial antibody to human leukocyte elastase was used in ELISA to demonstrate the serologic identity of elastase with the elastolytic material purified by 14-30 from $CD4^+$ T cells (Fig. 2A) or by 14-12 from $CD8^+$ T cells (data not shown). These results indicate that an elastase-like protease is a product of both $CD4^+$ and $CD8^+$ T cells and is found both membrane-associated and in a cell-free form.

In ELISA the monoclonal antibody 14-12 was immunoreactive with porcine pancreatic elastase (Fig. 2B) and human leukocyte elastase (data not shown). This monoclonal antibody is immunoreactive with factors of $CD8^+$, but not $CD4^+$ T cells, implying that there may be structural differences in the elastase complex or the elastase

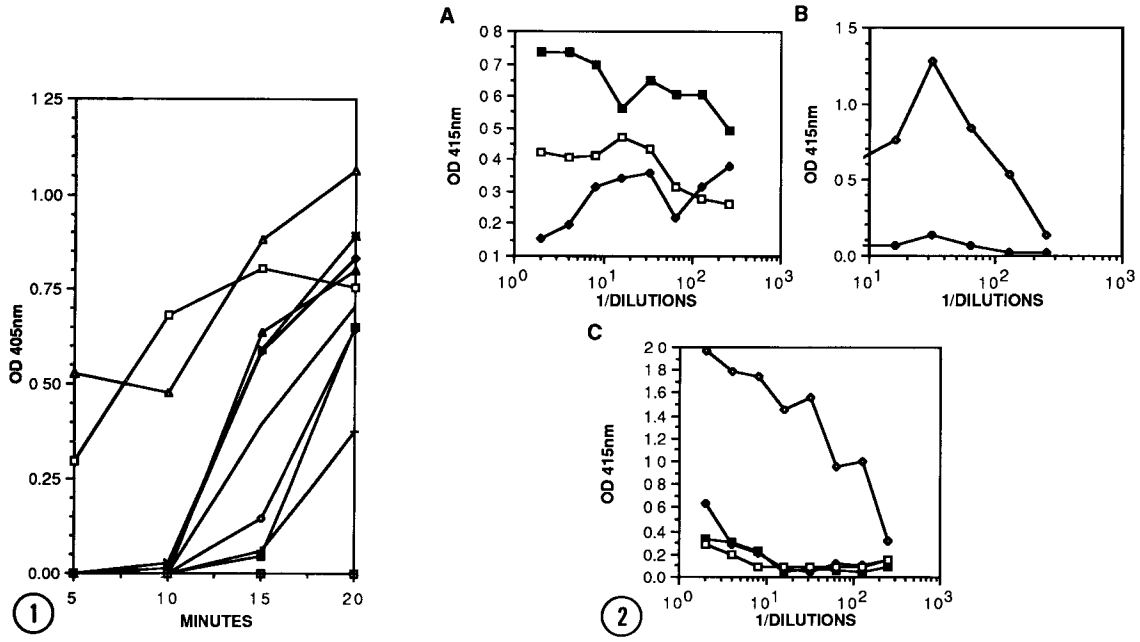

Fig. 1. Detection of elastolytic activity in T cell immunoregulatory factors. (♦) A1.1 purified on anti-T cell receptor; (∗) A1.1 purified on the poly-18 antigen; (■) AF5.C6 (0.743 mg/ml); (□) F12.23 (3.51 mg/ml); (●) 34s-704 membranes or (◇) purified on the immunizing antigen; (+) Ts3-3KS-21; (△) porcine pancreatic elastase (5 μg/ml); (▲) human leukocyte elastase (5 μg/ml), and (⊞) F12.23 from cells co-cultured in the presence of N-t-Boc-L-Alanyl-Prolyl-Nvaline p-chloro thiobenzyl ester (BAPV, Sigma), a synthetic substrate of leukocyte elastase.

Fig. 2. Serological identification of elastase and α_1-PI produced by CD4$^+$ and CD8$^+$ cells in ELISA. A. Dilutions of 51H7D unfractionated culture supernatant (♦), 51H7D purified culture supernatant (□), or 51H7D purified membrane extracts (■) incubated with sheep anti-human leukocyte elastase. B. Dilutions of porcine pancreatic elastase incubated with monoclonal antibody 14-12 (◇) or 14-30 (♦) ascites fluid (1/100). C. (◇) Dilutions of D10.G4.1, (■) 51H7D, (♦) Ts3-3KS-21, and (□) human leukocyte elastase incubated with anti-α_1-PI.

molecule itself in these cell types. Elastase is often found in association with its natural inhibitor α_1-PI (17). By ELISA, α_1-PI was detected in material purified on 14-12 and 14-30 from both CD4$^+$ and CD8$^+$ T cells (Fig. 2C).

By Western blot analysis of material purified on 14-12 and 14-30 from both CD4$^+$ and CD8$^+$ T cells, bands were detected which were cross-reactive with commercial antibodies to human leukocyte elastase (Fig. 3A). Elastase was detected in fluid-phase complexes with approximate molecular mass 80K, 72K, 60K, 30K, 27K, and 25K and in membrane-associated complexes with molecular mass 63K and 58K. Because of the inability to detect elastase in its enzymatically active form in factors from T cells, it is probable that the elastase detected in Western blot analysis is in its proenzyme form with molecular weight 30K, in its activated form with molecular weight 27K or 25K, and complexed with other components in the higher molecular weight bands. The molecular

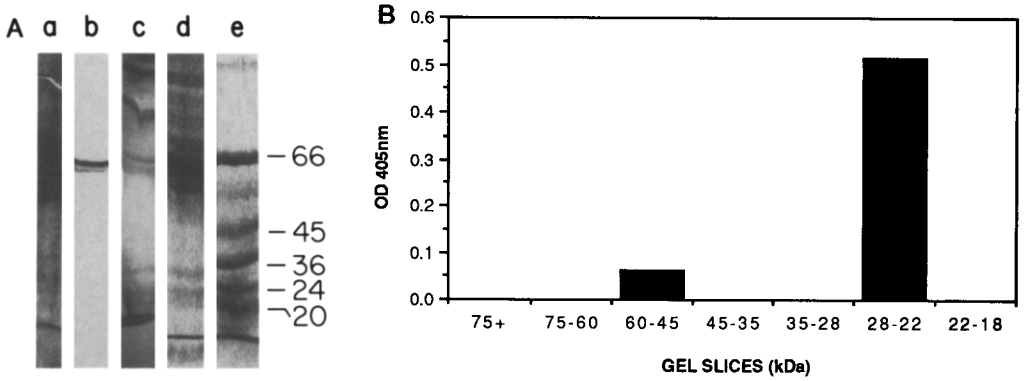

Fig. 3. Western blot of purified elastase from a T hybridoma. A. After electrophoresis, material from 51H7D were either stained with (a) periodic acid-Schiff stain, (d) Coomassie blue R-250, or transferred to Immobilon by Western blotting and incubated with (b) monoclonal anti-human α_1-PI, or (c) anti-human leukocyte elastase. Molecular weight markers (e) are expressed as kilodaltons. B. Molecular weight of material exhibiting elastolytic activity.

weight of the specific component responsible for imparting elastolytic activity was determined. Material was fractionated by size in preparative SDS-PAGE under reducing conditions, and the material extracted from the individual gel slices was used in an assay measuring elastolytic activity. Material with approximate molecular mass 28-22K evidenced elastolytic activity (Fig. 3B).

Upon binding elastase, a 4K molecular weight peptide is cleaved from the N-terminus of α_1-PI (18). The glycoprotein α_1-PI was demonstrted to be a component of the purified complex by Western blot analysis with molecular weight 58K and 54K (Fig. 3A). When the carbohydrate content of purified material was analyzed, bands identified as 56K, 26K, 22K, and 20K were found to contain carbohydrates by periodic acid-Schiff stain (Fig. 3A). Bands at 49K did not appear to be glycosylated.

It has been found that certain cells exhibit elastase/α_1-PI receptors on their cell surfaces (19). Therefore, it was important to determine whether the elastase purified from T cells was synthesized by the T cells or absorbed from serum. Using in vitro translation of poly A+ RNA from two murine T hybridomas, increased elastolytic activity was detected (Fig. 4A). That elastase is a lymphokine synthesized by T cells has not before been recognized and may represent a potentially powerful mechanism of regulation of T cell activation. By polymerase chain reaction (PCR), an appropriately sized cDNA fragment (0.65kb) has been amplified from several murine T cell hybridomas (Fig. 4B). The gene is currently being cloned for sequencing.

DISCUSSION: Elastase is a serine protease originally described to be secreted by pancreatic cells (20), but investigators have now found that elastase is produced by

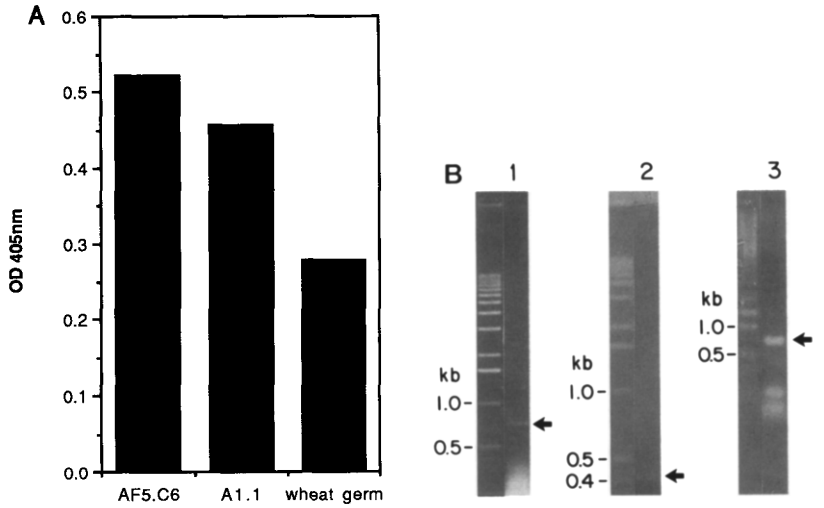

Fig. 4. In vitro translation of cDNA and PCR analysis of T hybridomas. A. Elastolyic activity in translated products from AF5.C6 and A1.1 as compared to wheat germ. B. Agarose gel for analysis of PCR with RNA from AF5.C6 (Lane 1), A1.1 (Lane 2), and Ts1-29 (Lane 3). Corresponding RNA ladders represent kilobases as indicated.

neutrophils (21) and macrophages (22) with differing characteristics. In addition to its many other important functions, previously characterized elastase has a wide spectrum of substrates and immunologically active ligands including elastin, collagen, fibronectin, cartilage proteoglycan, heparin, insulin, and Complement components C3, C5, C1 esterase inhibitor, and Factor B, in addition to IgG, kappa light chains (rev. 23), and CD4 (24). Elastase has been found to interact with immunocompetent cells by increasing proliferation of lymphocytes, modulating lymphocyte membrane receptors, and inhibiting monocyte chemotactic activity (rev. 23).

Furthermore, elastase has been found to be involved in a number of immunologic disease states, including rheumatoid arthritis, primary amyloidosis, Wegener's granulomatosis, cystic fibrosis, atherosclerosis, leukemia, systemic lupus erythematosus, glomerulonephritis, acute hemorhagic pancreatitis, periodontitis, emphysema, and in the sequelae following severe burn injury or other trauma (rev. 23). It was previously thought that the elastase found in association with these diseases was derived from macrophages or neutrophils. There is now reason to believe that T cell derived elastase itself may be directly involved in some of these disease processes.

The natural inhibitor of human elastase, α_1-PI, has been determined to have more than 75 allelic variants and many more isoforms due to variation in both quantity and type of N-linked and O-linked glycosylation. This ubiquitous protein has been found to interact with several molecules of immunological importance including clotting factors X and XI, thrombin, plasmin, kallikrein, trypsin, and chymotrypsin, as well as collagenase, Cathepsin

G (25), IgA and kappa light chains (covalently), C3, fibrinogen, and myeloperoxidase (rev. 23). In addition, α_1-PI has been shown to suppress immunoglobulin synthesis, lymphocyte proliferation, and phagocytosis by monocytes (26). As elastase, α_1-PI has been implicated in several disease processes including emphysema, atherosclerosis, respiratory distress syndrome, rheumatoid arthritis, panniculitis, pre-eclampsia, noninfectious hepatitis, cirrhosis, vasculitis, cold urticaria, and even twinning (26).

The association of elastase and α_1-PI with T cells raises the possibility that these molecules are themselves involved in regulatory mechanisms of the immune response. The possible regulatory role of differentially glycosylated molecules, including elastase and α_1-PI in determining immune responsiveness may be controlled by a) the specific membrane receptor on the macrophage, B or T cell to which the elastase/α_1-PI complex is targeted, b) the substrate specificity of the elastase component, c) the isoform of the polymorphic elastase inhibitor α_1-PI, or d) the proximity of other molecules associated with the function of this complex.

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